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Nanocomposites of bacterial cellulose/hydroxyapatite for biomedical applications

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Abstract

In the present work, a nanocomposite material formed by bacterial cellulose (BC) networks and calcium-deficient hydroxyapatite (HAp) powders was synthesized and characterized. The HAp nanoparticles were previously prepared by a wet chemical precipitation method, starting from aqueous solutions of calcium nitrate and di-ammonium phosphate salts. Energy-dispersive spectroscopy reveals that the prepared HAp corresponds to calcium-deficient hydroxyapatite. BC-HAp nanocomposites were prepared by introducing carboxymethylcellulose (CMC) into the bacteria culture media. HAp nanoparticles were then introduced and remained suspended in the culture medium during the formation of cellulose nanofibrils. The maximum gel thickness was obtained after 21 days of bacteria cultivation. X-ray diffractograms showed the difference of crystallinity among the materials involved in the formation of nanocomposites. The inorganic and organic bonds that corresponded to hydroxyapatite and bacterial cellulose respectively, were depicted by attenuated total reflectance Fourier transform infrared spectra. Scanning electron microscopy and atomic force microscopy measurements confirmed the formation of networks and fibres with smaller diameter corresponding to BC synthesized in the presence of CMC. Image analysis was also used to assess the orientation distributions and Feret diameters for networks of BC and BC-CMC. Thermogravimetric analysis showed that the amount of the mineral phase is 23.7% of the total weight of the nanocomposite. Moreover, HEK cells were cultivated and the biocompatibility of the materials and the cell viability was demonstrated.
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1. Introduction

Bacterial cellulose (BC) is a polysaccharide used traditionally in the food industry [1,2], later in the fabrication of reinforced paper [3] and recently it was investigated as a material for medical applications. Studies carried out in vitro and in vivo have demonstrated its biocompatibility [4,5]. Due to its good mechanical properties, water sorption capacity, porosity, stability and conformability, BC has been used in tissue engineering of cartilage [6], replacement of blood vessels in rats [7] and in the wound healing process [8,9].

BC is pure cellulose with no other components [1]. Nanocomposites based on BC can be fabricated statically either by using the synthesized BC gel or modifying the cellulose biosynthesis. For instance, BC nanocomposites for biomedical applications with improved mechanical properties were created by soaking BC on polyacrilamide and gelatin solutions [10,11]. BC-hydroxyapatite scaffolds for bone regeneration have been developed by immersing the BC gel in simulated body fluid (SBF) or in both calcium and phosphate solutions [12–16]. Furthermore, BC-polyester and BC-PVA nanocomposites were developed for potential applications as vascular implants [17,18].

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Some researchers have introduced different materials into the culture media of BC. BC synthesized in the presence of collagen [19] and chitosan [20] has improved properties as wound dressing and for other biomedical applications. It has been reported that BC membranes produced in the presence of carboxymethylcellulose (CMC) have better adsorption capacity of metal ions than membranes of pure BC [21–23].

However, the addition of some polymers can modify drastically the cellulose biosynthesis. The addition of CMC into the culture medium alters the crystallization and assembly of the cellulose fibrils [24]. A similar effect occurred when polyethylene oxide is added to the medium in the process for obtaining BC based nanocomposites [25].

In agitated cultures, it has been demonstrated that BC can be produced in the presence of solid particles (i.e. glass beads, paper fibres and $CaCO_3$) without affecting the rate of formation of the hydrogel [26]. Recent studies report the inclusion of silica particles of 10–20 nm and multi-walled carbon nanotubes (20–40 nm outer diameter, 10–50 μm length) into the culture medium to produce BC-nanocomposites in static cultures [27,28]. Hydroxyapatite (HAp) has been used in bone regeneration and as a substitute of bone and teeth because it is a biocompatible, bioactive, non-inflammatory, non-toxic and non-immunogenic material [29,30].

The aim of this study was to fabricate BC-HAp nanocomposites by the formation of cellulose nanofibrils in the presence of a mineral phase in a static culture. In order to suspend the HAp nanoparticles, the bacteria culture media were modified by the addition of CMC. Nanocomposites were characterized by means of X-ray diffraction (XRD), Fourier transform infrared spectroscopy (ATR-FTIR), scanning electron microscopy (SEM), atomic force microscopy (AFM), thermogravimetric analysis (TGA) and image analysis. In vitro biocompatibility and viability was assessed using HEK cells.

2. Materials and methods

2.1. Preparation of HAp powders

Hydroxyapatite (HAp) powders were prepared in vitro using a wet chemical precipitation method. $Ca(NO_3).4H_2O$ and $(NH_4)_2HPO_4$ were used as Ca and P precursors respectively, following the next basic reaction:

$$5Ca(NO_3).4H_2O + 3(NH_4)_2HPO_4 \rightarrow Ca_5(PO_4)_3OH + 6NH_4NO_3 + 19H_2O + 4HNO_3$$

Initially, 0.6 M $(NH_4)_2HPO_4$ and 1.0 M $Ca(NO_3).4H_2O$ solutions were adjusted at pH 10.2 by the addition of concentrated NH_4OH. The phosphate solution was added in drops into the stirring calcium solution at 70 °C. Stirring at this temperature was carried out for 24 h and this process was followed by further stirring for 48 h at room temperature. The resultant milky solution was filtered-washed four times with distilled water. The precipitate was dried in a vacuum oven at 60 °C for 24 h. The resultant HAp powders were milled in an agate mortar.

2.2. Preparation of BC and BC-HAp nanocomposite gels

The original culture medium for the growth of BC consisted of 1.0% (w/v) D-glucose, 1.5% (w/v) peptone, 0.8% (w/v) yeast extract and 0.3% (v/v) glacial acetic acid. The pH of the solution was adjusted to 3.5 with hydrochloric acid. In order to maintain the medium free of the action of microorganisms, it was autoclaved at 121 °C for 20 min. After the medium had cooled down, 0.01% (w/v) cycloheximide and 0.5% (w/v) absolute ethanol were added. Cycloheximide was used in order to avoid the presence of filaments while ethanol acts as an additional energy source for ATP generation enhancing thus the BC production in stationary cultures [31]. The described culture medium was used by Lisdiyanti et al. [32] and Yamada et al. [33] for the identification of acetic acid bacteria.

The strain *Gluconacetobacter saccharivorans* (LMG 1582) isolated from a Kombucha tea mat [34] was inoculated and cultivated at 30 °C for 21 days. After this period, BC gels were removed and washed with deionized water. In order to remove bacteria and eliminate the remaining culture medium, the cellulose pellicles were boiled in 1.0 M NaOH at 70 °C for 90 min followed by repetitive rinsing in deionized water.

For the formation of the new nanocomposite, HAp nanoparticles were suspended in the culture medium. In order to avoid the settling of HAp nanoparticles, the viscosity of the solution was controlled using carboxymethylcellulose sodium salt (CMC) from Acros Organics (average MW 25,0000, DS = 1.2). CMC was added to the medium in concentrations of 1.0 and 2.0% (w/v) and stirring was carried out until CMC dissolved. HAp powders were added in each vessel in concentrations of 1 and 2% (w/v) and the solutions remained under agitation overnight at room temperature. The pH was adjusted to 3.5. Both cycloheximide and ethanol were added after the sterilization process to prevent ethanol from reaching the boiling point as well as the melting of cycloheximide. Finally, an inoculum of a previously cultivated BC was introduced in the cultures. The culture conditions and washing process were the same as described above.

2.3. Samples preparation

In order to characterize the nanocomposite structures, water was removed from gels by either freeze drying, solvent exchange or hot pressing.

Pure BC, BC synthesized in the presence of 1% w/v CMC in the culture medium (BC-CMC), and nanocomposites of BC-CMC with HAp added to the culture medium in concentration of 1% w/v (BC-CMC-HAp) were frozen in liquid nitrogen (−196 °C) and freeze-dried in a Telstar

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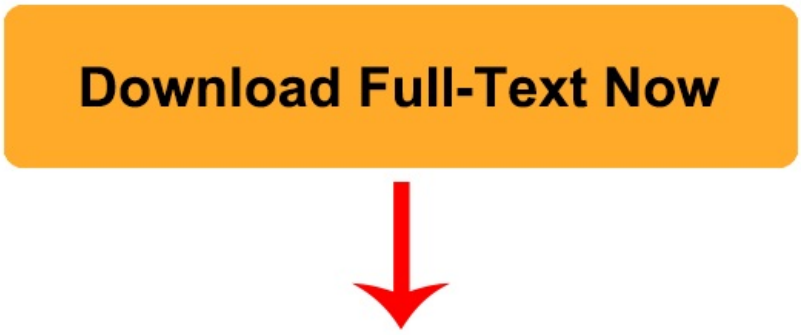

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